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DNA Adduct Formation in Relation to Lymphocyte Mutations and Lung Tumor Induction in F344 Rats Treated with the Environmental Pollutant 1,6-Dinitropyrene

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Epidemiological studies suggest an association between exposure to diesel emissions and an increased incidence of lung and bladder cancer in humans. Of the compounds associated with diesel emissions, 1,6-dinitropyrene is a particularly potent mutagen and carcinogen. In these experiments we administered [4,5,9,10-^{3}H]1,6-dinitropyrene (30 or 100 μg) directly to the lungs of F344 rats according to a protocol known to induce lung tumors and characterized the DNA adducts present in the target tissue. In addition, we examined the adducts present in spleen lymphocytes and assayed for the induction of mutations at the hypoxanthine-guanine phosphoribosyltransferase locus in these cells, as measured by the frequency of 6-thioguanine-resistant (TG^r) T-lymphocytes. Adduct formation was detected in both lung and spleen lymphocyte DNA, with the extent of binding being dose-dependent in the lymphocytes but not the lung. ^{32}P-Postlabeling analyses indicated the formation of a major DNA adduct, *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene, in both tissues. 1,6-Dinitropyrene treatment resulted in a dose-dependent increase in TG^r T-lymphocytes, with the increase being detected for at least 21 weeks after treatment. These data indicate that 1,6-dinitropyrene is metabolically activated by nitroreduction to form DNA adducts in both the target tissue and spleen lymphocytes and that a tumorigenic dose results in a significant induction of TG^r T-lymphocytes.

Introduction

Nitropolycyclic aromatic hydrocarbons (nitro PAHs) are widespread environmental contaminants resulting from a number of combustion processes (*1*). A particularly notable source of these compounds is diesel exhaust, which induces lung tumors in experimental animals and has been associated with the induction of lung and urinary bladder cancer in humans (*2*). Although more than 50 nitro PAHs have been detected in diesel engine emissions, much emphasis has been placed on the nitropyrenes. 1-Nitropyrene, which is the predominant nitropyrene found in diesel particle extracts, is mutagenic in bacterial and mammalian cells and tumorigenic in rats and mice (*2,3*). Dinitropyrenes are detected at much lower concentrations than 1-nitropyrene; however, these compounds, in particular 1,6- and 1,8-dinitropyrene, are exceedingly potent bacterial mutagens and, when tested in the same animal model, are much more tumorigenic than 1-nitropyrene (*3*). These findings suggest that dinitropyrenes may be important markers for assessing the risk associated with exposure to diesel emissions.

The metabolic activation of dinitropyrenes involves nitroreduction to *N*-hydroxyamino intermediates followed by *O*-acetylation to give *N*-acetoxy derivatives that will react with DNA (*3*). DNA adducts resulting from the nitroreduction and *O*-acetylation of 1,6- and 1,8-dinitropyrene have been characterized and detected in bacteria and experimental animals. These adducts are presumably responsible for the mutagenic and tumorigenic responses observed with the dinitropyrenes.

Although DNA adducts are important biomarkers for carcinogen exposure, they have a finite lifetime due to DNA repair and/or cell turnover. Thus, additional techniques allowing assessment of more-than-recent exposures should clearly be useful. One such approach has been to assay for antibodies to DNA adducts in workers exposed to high levels of PAHs (*4*). An

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alternative technique is to determine if carcinogen exposure induces lymphocyte mutations because, if mutations occur in stem cell populations, they may be detected for periods long after the carcinogen exposure (*5*). Additional advantages of the latter approach are that it provides a biological assessment of the impact of particular DNA adducts and that the types of mutations may be compound specific, making it possible to obtain a fingerprint of DNA sequence changes caused by carcinogen exposures.

In these experiments, we administered 1,6-dinitropyrene directly to the lungs of F344 rats according to a protocol (*6*) known to induce lung tumors and characterized the DNA adducts present in the target tissue. In addition, we examined the adducts present in spleen lymphocytes and assayed for the induction of mutations at the hypoxanthine-guanine phosphoribosyltransferase (*hprt*) locus in these cells.

Materials and Methods

DNA Adduct Analyses

Male F344 rats (12 weeks old; obtained from the breeding colony at the National Center for Toxicological Research, Jefferson, AR) were anesthetized with a mixture of ketamine and xylazine (*7*) and subjected to a left lateral thoracotomy. [4,5,9,10-^{3}H] 1,6-Dinitropyrene (0, 30, or 100 μg; 767 or 1059 mCi/mmole) in 50 μL of beeswax and tricaprylin (1:1) was then administered using the lung implantation method of Stanton et al. (*8*) as described by Iwagawa and co-workers (*6*). Three, 7, 14, and 28 days after treatment, two or three animals were exposed to carbon dioxide, decapitated, and the lungs and spleens were quickly excised. Lung nuclei were prepared by the method of Basler et al. (*9*), and spleen lymphocytes were isolated by the technique of Aidoo et al. (*10*). DNA was extracted from the nuclei and cells by slight modifications of the method reported in Beland et al. (*11*). The DNA was quantified by UV spectrometry, and the extent of adduct formation was determined by liquid scintillation counting.

To characterize the adducts present in lung nuclei and spleen lymphocytes, additional aliquots of DNA were analyzed by ^{32}P-postlabeling, which was conducted as described in Smith et al. (*12*).

Analysis of Mutation Induction at the *hprt* Locus of Spleen Lymphocytes

Additional rats were treated as described above with 0, 30, or 100 μg 1,6-dinitropyrene or injected with 40 mg *N*-ethyl-*N*-nitrosourea (ENU; 150 mg/ kg body weight). The latter group served as a positive control for the mutagenesis assays. Three, 9, 12, 15, and 21 weeks after surgery, two solvent-treated control rats, two 1,6-dinitropyrene-treated rats, and one ENU-treated rat were euthanized by exposure to carbon dioxide. Their spleens were removed aseptically and lymphocytes were isolated and pooled by group. The number of T-lymphocytes with mutations at the *hprt* locus, as evidenced by growth of the lymphocytes in the presence of the purine analog 6-thioguanine, was determined by the limiting dilution clonal assay described in Aidoo et al. (*10*), modified by using conditioned medium as a source of T-cell growth factor (Aidoo et al., manuscript in preparation).

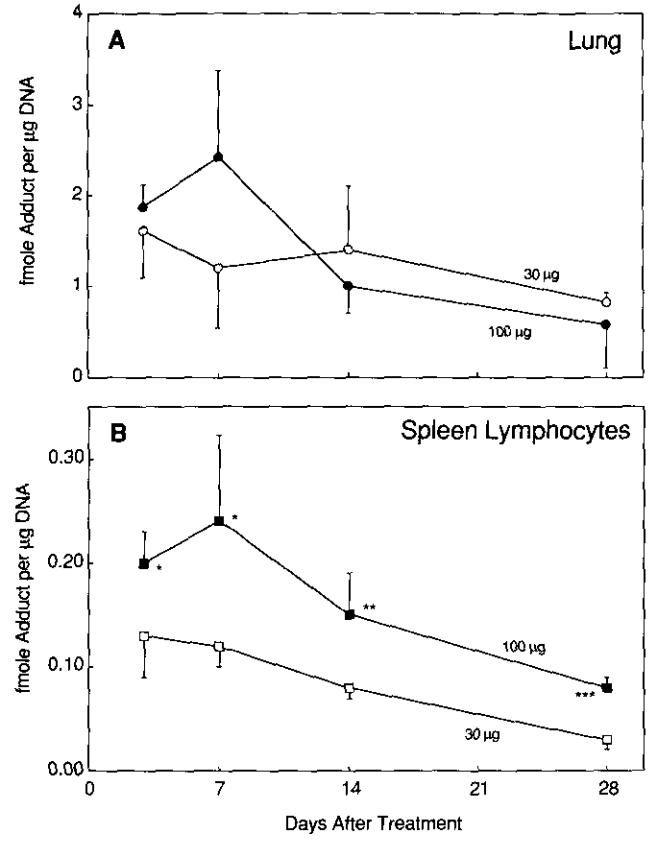

FIGURE 1. Binding of 1,6-dinitropyrene to (*A*) lung nuclei and (*B*) spleen lymphocyte DNA. [4,5,9,10-^{3}H]1,6-Dinitropyrene (30 or 100 μg) was administered directly into the lungs of male F344 rats. At the times indicated, the rats were killed, lung nuclei and spleen lymphocytes were prepared, DNA was isolated, and the extent of binding was quantified by UV and liquid scintillation spectrometry. The data are presented as means ± SD of three rats, except for the 28-day point, which only had two rats. Asterisks indicate that DNA binding in the 100-μg group was significantly greater than the 30-μg group: $^*p < 0.1$; $^{**}p < 0.05$; and $^{***}p < 0.01$.

Results

Analysis of the Binding of 1,6-Dinitropyrene to Lung and Spleen Lymphocyte DNA

The extent of binding of 1,6-dinitropyrene to lung DNA after lung implantation is shown in Figure 1*A*. At each sampling point, similar values were found with both doses. With the 100-μg treatment, maximum binding occurred 7 days after treatment, while with the 30-μg dose the highest level of binding was found 3 days after dosing. With both treatments, there was a slow decrease in the binding such that by 28 days after dosing binding had decreased to 25–50% of the peak values.

The magnitude of adduct formation with spleen lymphocyte DNA (Fig. 1*B*) was considerably lower than that found with lung DNA (Fig. 1*A*). As with lung DNA, the binding to spleen lymphocyte DNA reached a maximum at 7 days after treatment for the 100-μg dose and 3 days after dosing for the 30-μg group. In contrast to lung DNA, there was a significant dose response in

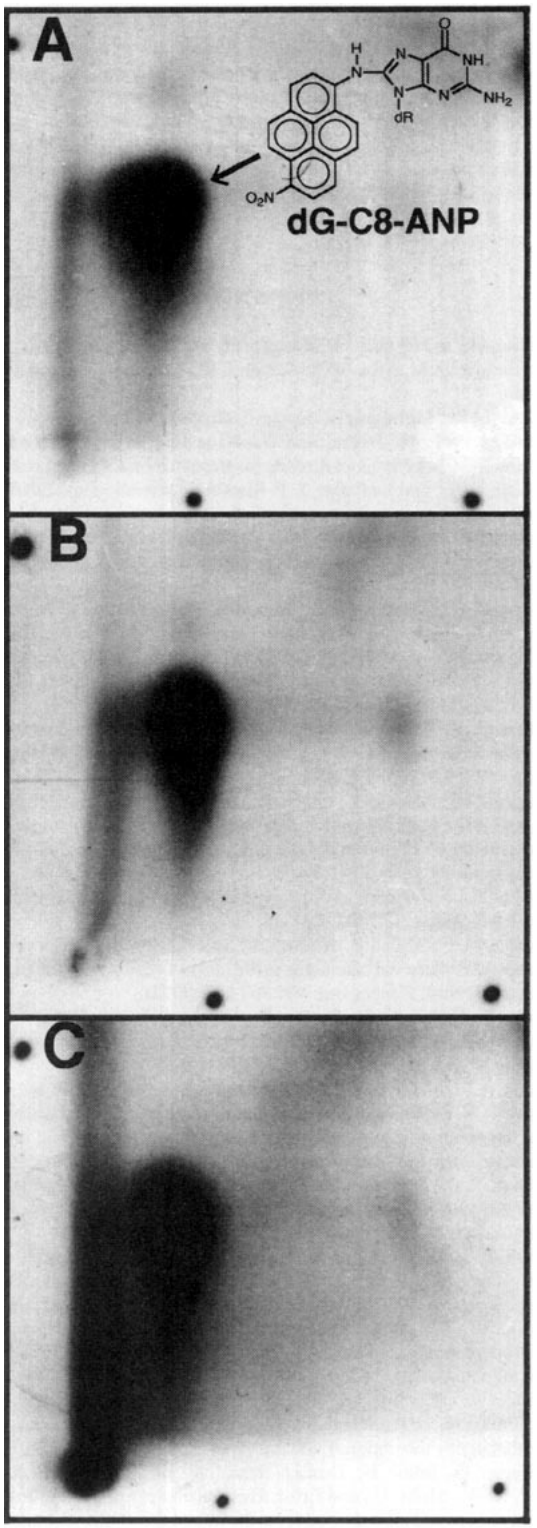

FIGURE 2. ^{32}P-Postlabeling autoradiographs of (*A*) DNA standard modified with *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene (dG-C8-ANP), (*B*) lung nuclei from a male F344 rat treated with 100 μg [4,5,9,10-^{3}H]1,6-dinitropyrene and killed after 7 days, and (*C*) spleen lymphocyte DNA from a male F344 rat that was treated with 30 μg [4,5,9,10-^{3}H]1,6-dinitropyrene and sacrificed after 3 days.

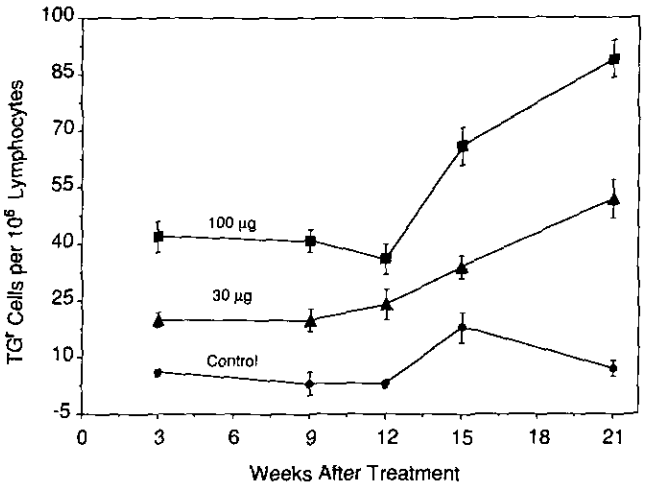

FIGURE 3. Mutation induction in spleen lymphocytes of male F344 rats treated with solvent or 30 or 100 μg 1,6-dinitropyrene. Rats were given a single dose of the compound indicated, killed after 3, 9, 12, 15, or 21 weeks, and the number of 6-thioguanine-resistant (TG^r) spleen T-lymphocytes was determined by means of a limiting dilution clonal assay. At each time point, the response in the 100-μg group was significantly greater than the 30-μg group ($p < 0.01$).

the binding to spleen lymphocyte DNA, with the binding in the 100-μg dose group being 1.5- to 2.7-fold greater than the 30-μg group at each time point. Twenty-eight days after treatment, the binding had decreased to about 30% of the peak values.

Aliquots of lung and spleen lymphocyte DNA were assayed by ^{32}P-postlabeling (Fig. 2). In each instance, a single major adduct was detected that had the same elution characteristics as the adduct obtained from reacting *N*-hydroxy-1-amino-6-nitropyrene with DNA. This adduct has been characterized as *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene (*13*).

Mutation Induction at the *hprt* Locus of Spleen T-Lymphocytes

Mutations were assayed 3, 9, 12, 15, and 21 weeks after lung implantation of 0, 30, or 100 μg of 1,6-dinitropyrene. As shown in Figure 3, there was a significant increase in 6-thioguanine-resistant (TG^r) T-lymphocytes compared to the solvent-treated controls, with the 100-μg dose of 1,6-dinitropyrene typically giving 2-fold more TG^r T-lymphocytes than the 30-μg treatment. The extent of induction was relatively constant for the first 9–12 weeks and then increased with each subsequent sampling.

The direct-acting mutagen, ENU, injected IP at a concentration of 150 mg/kg body weight, induced between 189 and 387 TG^r T-lymphocytes per 10^6 cells at each interval ($p < 0.001$ compared to control rats; data not shown).

Discussion

Iwagawa et al. (*6*) demonstrated that the administration of 1,6-dinitropyrene, a component of diesel emissions, by the lung implantation method of Stanton et al. (*8*) results in a dose-dependent induction of lung tumors in male F344 rats, with 30 and 100 μg producing 40 and 80% incidences, respectively. Using their protocol, we found that 1,6-dinitropyrene is metabo-

lically activated by nitroreduction, presumably to *N*-hydroxy-1-amino-6-nitropyrene, and that this latter compound either directly or after subsequent activation reacts with DNA in the target tissue to give *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene. Although these doses of 1,6-dinitropyrene give markedly different tumor incidences, a difference was not observed in the binding to lung DNA as a function of dose. This may reflect the fact that the compound is localized within a beeswax pellet in a small portion of the lung, and yet the adduct analyses were conducted with DNA from the entire tissue. In contrast to lung DNA, a dose-dependent formation of *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene was found in spleen lymphocyte DNA, with the 3.3-fold increase in dose resulting in approximately a 2-fold increase in adduct concentration. The 2-fold difference in adduct concentration correlated with a 2-fold difference in the frequency of TG^r T-lymphocytes between 30 and 100 μg 1,6-dinitropyrene. This suggests that mutations are being induced by 1,6-dinitropyrene treatment and that these mutations are the direct result of the formation of *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene in spleen lymphocyte DNA. This interpretation needs to be confirmed by molecular analyses of the DNA alterations in the putative mutants.

In both the lung and spleen lymphocyte DNA, the concentration of *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene decreased by 50–75% during a 1-month period after treatment. In contrast, the frequency of TG^r T-lymphocytes remained relatively constant for approximately 9–12 weeks and then began to increase. Thus, lymphocyte mutations may be a longer-lived biomarker than DNA adducts for assessing previous exposures to genotoxic agents, such as nitro PAHs.

Lymphocyte mutation assays coupled with DNA adduct measurements may also be important tools for determining the risk associated with exposure to mixtures. Diesel emissions, for example, have been associated with the induction of lung tumors in both rats (*14*) and humans (*2*). A comparison of the DNA adducts obtained from exposing rats to diesel exhaust extracts (*15*) and 1,6-dinitropyrene should allow a determination of the relative contribution of 1,6-dinitropyrene to the tumorigenic response. Molecular analysis of the mutations induced by 1,6-dinitropyrene in the rat model may indicate a fingerprint for mutation induction in terms of the types and location of mutations in the *hprt* gene. If 1,6-dinitropyrene contributes to the tumorigenic response observed with diesel emissions, this mutational fingerprint should also be found in animals treated with diesel exhaust extracts. Mutation assays could then be conducted in humans suspected of being exposed to high levels of diesel emissions. An increase in TG^r T-lymphocytes would provide a biological assessment of the impact of this exposure, and molecular analysis of the mutants would provide evidence that the biological response was indeed due to exposure to diesel emissions.

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